JPn Neuroscience

The Journal of Physiology

J Physiol 590.10 (2012) pp 2427–2442

Investigation of the neuroanatomical substrates of reward seeking following protracted abstinence in mice

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Key points

- Persistent vulnerability to relapse represents a major challenge in the treatment of drug addiction. The brain circuitry that underlies relapse-like behaviour can be investigated using animal models.
- This study compared the brains of mice that had relapsed to morphine with mice that had relapsed to sucrose following abstinence. We found that while some brain regions were implicated in both drug and food seeking, other specific parts of the brain were activated for either sucrose or morphine relapse.
- Common regions included those with established involvement in reward and relapse-like behaviour. In addition we found some regions not previously linked to these behaviours.
- Overall, while our findings support existing literature regarding relapse-like behaviour in rats, we have additionally identified brain regions outside this established circuitry which are worthy of further investigation.

Abstract Persistent vulnerability to relapse represents a major challenge in the treatment of drug addiction. The brain circuitry that underlies relapse-like behaviour can be investigated using animal models of drug seeking. As yet there have been no comprehensive brain mapping studies that have specifically examined the neuroanatomical substrates of cue-induced opiate seeking following abstinence in a mouse operant paradigm. The aim of this study was to compare the brain regions involved in sucrose *vs.* morphine seeking following protracted abstinence in mice. Male CD1 mice were trained to respond for either sucrose (10% w/v) or intravenous morphine (0.1 mg kg^{-1} per infusion) in an operant paradigm in the presence of a discrete cue. Once stable responding was established, mice were subjected to abstinence in their home cages for 3 weeks and then perfused for tissue collection, or returned to the operant chambers to assess cue-induced reward seeking before being perfused for tissue collection. Brain tissue was processed for Fos immunohistochemistry and Fos expression was quantified in a range of brain nuclei. We identified unique patterns of neuronal activation for sucrose and morphine seeking mice as well as some overlap. Structures activated in both 'relapse' groups included the anterior cingulate and orbitofrontal cortex, nucleus accumbens shell, bed nucleus of the stria terminalis, substantia nigra pars compacta, ventral tegmental area, hippocampus, periaqueductal grey, locus coeruleus and lateral habenula. Structures that were more activated in morphine seeking mice included the nucleus accumbens core, basolateral amygdala, substantia nigra pars reticulata, and the central nucleus of the amygdala. The dorsal raphe was the only structure examined that was specifically activated in sucrose seeking mice. Overall our findings support a cortico-striatal limbic circuit

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DOI: 10.1113/jphysiol.2011.225219

driving opiate seeking, and we have identified some additional circuitry potentially relevant to reward seeking following abstinence.

(Received 24 November 2011; accepted after revision 1 March 2012; first published online 5 March 2012)
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Abbreviations AC, anterior cingulate cortex; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; CPP, conditioned place preference; CRF, corticotropin-releasing factor; DG, dentate gyrus; FR1, fixed ratio of 1; IEG, immediate early gene; IL, infralimbic cortex; LC, locus coeruleus; MCH, melanin-concentrating hormone; NAc, nucleus accumbens; NGS, normal goat serum; OFC, orbitofrontal cortex; PAG, periaqueductal grey; PL, prelimbic cortex; PVA, periventricular nucleus of the thalamus; PVN, paraventricular nucleus of the hypothalamus; SN, substantia nigra; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; VP, ventral pallidum; VTA, ventral tegmental area.

Introduction

A major challenge in the treatment of drug addiction lies in preventing relapse, a risk that remains high even following protracted abstinence (O'Brien, 1997). Repeated drug use results in the formation of learned associations between the drug experience and various stimuli associated with drug taking. Exposure to these drug-associated cues can evoke powerful cravings in addicted individuals, which can ultimately lead to relapse (O'Brien *et al.* 1998; Childress *et al.* 1999).

Relapse-like behaviour can also be observed in animals upon exposure to drug-associated cues (Meil & See, 1996). The reinstatement model is a preclinical model commonly used to investigate the neurobiology that underlies cue-induced relapse to drug seeking (Shaham *et al.* 2003). Using this paradigm, animals (usually rodents) are trained to perform an operant task such as a nose poke or lever press in order to obtain a reward, which can be either a drug or a natural reinforcer. Reward delivery can be paired with discrete, contextual or discriminative cues. Once the operant behaviour has been acquired it can then be extinguished by removal of both reward reinforcement and associated cues. Following extinction, operant responding can be reinstated by exposing the animal to the reward-associated cues.

Extinction training is a form of inhibitory learning, and it is therefore loosely analogous to humans participating in a formal rehabilitation programme. However in reality only a very small proportion of drug addicts participate in any formal rehabilitation. For example, in 2009 out of 23.5 million Americans who were considered to have an illicit drug or alcohol use problem, only 2.6 million (11.2%) received treatment at a specialty facility (SAMHSA, 2010). Therefore other relevant models of relapse are required that measure drug seeking following a period of abstinence (Reichel & Bevins, 2009).

Experiments investigating the circuitry underlying drug seeking following both abstinence and reinstatement broadly support a cortico-striatal limbic circuit driving drug seeking behaviour (Neisewander *et al.* 2000; Kalivas & Volkow, 2005; Di Ciano *et al.* 2008; Kuntz *et al.* 2008; Zavala *et al.* 2008; Steketee & Kalivas, 2011; Ziolkowska *et al.* 2011). These experiments typically involve either lesioning/reversible inactivation of discrete brain structures, or examination of immediate early gene (IEG) expression, which is indicative of neuronal activity (Herdegen & Leah, 1998). Although the majority of this research has been performed with respect to cocaine seeking, relapse to opiate seeking appears to involve similar circuitry with some additional structures identified (Kuntz *et al.* 2008; Rogers *et al.* 2008; Kuntz-Melcavage *et al.* 2009; Badiani *et al.* 2011). Experiments comparing drug seeking following abstinence *vs.* extinction have also revealed that the underlying mechanisms are at least partially dissociable (Fuchs et al., 2006; Van den Oever et al., 2008; Lasseter et al., 2010; Van den Oever et al., 2010).

Despite the increasing use of genetically modified mice in addiction research, there have so far been no systematic brain mapping studies that have examined the neuroanatomical substrates of cue-induced opiate seeking in a mouse operant paradigm. Therefore the aim of the present study was to utilise the neuronal activity marker Fos to investigate the possible brain nuclei involved in cue-induced opiate seeking *vs*, cue-induced sucrose seeking following abstinence in mice. Fos is the protein product of the immediate-early gene (IEG) *c-fos* and is thought to be a marker of stimulus-induced brain activity (Dragunow *et al.* 1987).

Fos expression was quantified across an extensive range of nuclei in the brains of mice that had exhibited reward seeking behaviour compared to mice that remained abstinent. Importantly, the level of lever pressing during the 'relapse' session was similar for both reinforcers, which removes the potential confound of differential motor activity on brain activity. This allows for a valid comparison of Fos expression between mice seeking morphine and mice seeking sucrose.

Methods

Ethical approval

All experiments were performed in accordance with the Prevention of Cruelty to Animals Act 1986, under the guidelines of the National Health and Medical Research Council of Australia Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia.

Animals

Age matched adult male CD1 mice were used, and during the course of the experiments they were singly housed in standard mouse boxes with nesting material available. Mice were kept in a reverse 12 h light–dark cycle (dark 07.00–19.00 h) and had access to food and water *ad libitum*.

Experimental design

Mice were trained to self-administer either sucrose (10% w/v, $n = 19$) or morphine (0.1 mg kg^{-1} per infusion, I.V., $n = 21$) in an operant paradigm in the presence of discrete cues. Once mice had acquired stable responding self-administration was assessed over 5 days. Mice were then subjected to a 3 week abstinence period in their home cage and randomly assigned to either 'relapse' or 'withdrawal' groups. Relapse mice were subsequently reintroduced to the operant chamber for 1 h in order to assess cue-induced reward seeking under extinction conditions ($n = 17$ morphine, $n = 14$ sucrose). One hour after the drug seeking session ended, a random sample of these mice were anaesthetised (pentobarbitone, 80 mg kg^{-1}, I.P.) and transcardially perfused for tissue collection ($n = 5$ morphine, $n = 4$ sucrose). The 'withdrawal' mice were anaesthetised and transcardially perfused at the end of the 3 week withdrawal period ($n = 5$ for sucrose, $n = 4$ for morphine). This resulted in four experimental groups for immunohistochemical experiments; morphine 'relapse' ($n = 5$), sucrose 'relapse' ($n = 4$), morphine withdrawal ($n = 4$) and sucrose withdrawal ($n = 5$).

Operant self-administration apparatus

Self-administration sessions took place in mouse operant chambers which were enclosed in sound attenuation boxes and ventilated with fans (Med Associates, St Albans, VT, USA). The chambers were equipped with an active lever which was paired with delivery of a reward, and an inactive lever which resulted in no outcome when pressed. A stimulus light located directly above the active lever would illuminate upon completion of the required instrumental task to coincide with reward delivery. An olfactory cue was also present in the form of a cap containing a drop of vanilla essence which was positioned directly below the active lever.

Sucrose self-administration

Mice in the sucrose groups were trained to lever press in order to obtain 5 μl of a 10% sucrose solution, which was delivered to a receptacle for oral consumption. Self-administration sessions lasted for 2 h, on a fixed ratio of one lever press per delivery (FR1). Both active and inactive lever presses were recorded using the Med Associates software, and contacts to the receptacle were detected by a lickometer.

Surgery

Mice in the morphine groups were cannulated as previously described (Brown *et al.* 2009). Mice were anaesthetized with isoflurane (5% induction, 1.5–2% maintenance, Rhodia Organic Fine Ltd, Bristol, UK) and implanted with an indwelling intravenous catheter into the left jugular vein. Catheters were constructed from a 3.5 mm length of Silastic tubing (0.30 mm I.D. × 0.64 mm O.D., Dow Corning, MI, USA) attached to a 22 gauge needle which was bent in a U shape at right angles to the luer. The catheter was inserted 1 cm into the vein and secured in place with suture. The remaining tubing ran subcutaneously behind the ear to exit at the top of the head. The catheter port was attached to the skull with Loctite 454 instant adhesive (Loctite Australia Pty Ltd, Caringbah, Australia) and secured in place with dental cement (Vertex-Dental, Zeist, the Netherlands). Whilst still under anaesthesia the catheter was flushed with 0.02 ml 0.9% saline containing heparin (90 U ml^{-1}, CSL Ltd, Parkville, Vic, Australia) and neomycin sulphate (4 mg ml^{-1}, Delta Veterinary Laboratories, Somersby NSW, Australia). Mice were administered Meloxicam (0.1 ml of 5 mg ml^{-1} stock, I.P., Boehringer Ingelheim, Germany) for pain relief and placed under a heat lamp for recovery. Catheters were flushed daily with 0.02 ml, 10 U heparinised saline with antibiotic immediately before each session, and again with 0.02 ml, 90 U heparinised saline at the end of each session to maintain catheter patency. Catheters were periodically tested for patency by flushing with 0.02 ml of a ketamine/midazolam mixture (15 mg ml^{-1} ketamine, Parnell Laboratories, Alexandria, NSW, Australia + 0.75 mg ml^{-1} midazolam, Janssen Pharmaceuticals, Beerse, Belgium). If prominent signs of hypnosis were not apparent within 3 s the mouse was removed from the experiment.

Morphine self-administration

Morphine self-administration sessions commenced 48 h after surgery, and lasted for 2 h each day on an FR1

schedule of reinforcement (0.1 mg kg^{-1} per infusion), as previously described (Brown *et al.* 2009). When the active lever was pressed morphine hydrochloride (Glaxo Australia Pty Ltd, Boronia, Vic, Australia) dissolved in 0.9% saline was delivered intravenously via syringes mounted on injector pumps (Med Associates) which were connected via Bcoex-T22 polyethylene/polyvinyl chloride (PE/PVC) tubing to 22 gauge swivels (Instech Laboratories, Plymouth Meeting, PA, USA). The same tubing ran from the swivel to the intravenous catheter port on the mouse. The stimulus light would illuminate for 3 s to coincide with drug delivery and a 10 s time-out period ensued to avoid overdose. If the active lever was pressed during the time out period the light would not illuminate and no drug was infused. Responses on the active lever (both drug infusions and time outs) as well as responses on the inactive lever were recorded. If a maximum of 50 morphine infusions was reached in a 2 h session, the session was terminated and the mouse returned to the home cage.

Perfusion protocol/tissue preparation

Mice were anaesthetised using sodium pentobarbitone (80 mg kg^{-1}, 0.1 ml per 10 g body weight, I.P.) and transcardially perfused with 50 ml phosphate-buffered saline (PBS; 0.1 M, pH 7.4) followed by fixation with 50 ml 4% paraformaldehyde (PFA; Sigma-Aldrich, St Louis, MO, USA) in PBS. Mice were immediately decapitated, and the brains were dissected and postfixed overnight in 10% sucrose in 4% PFA. Using a cryostat brains were sliced as 40 μm coronal sections and floated in 48-well plates containing cryoprotectant solution (Watson *et al.* 1986) and stored at −20°C until use. In order to generate reference maps, every fourth section was transferred to a 48-well tissue plate containing PBS, slide-mounted with 0.5% gelatin and left to air dry overnight. Once dry the sections were counterstained with neutral red (0.5%, Sigma-Aldrich), serially dehydrated with ethanol, cleared with X-3B solvent and coverslipped with Depex Mounting Medium (BDH Laboratory Supplies, Poole, UK).

Diaminobenzidine (DAB) immunohistochemistry

Free floating sections were washed in 0.1 M PBS (3 × 10 min) then pre-blocked with 10% normal goat serum (NGS), 0.3% Triton X-100 and 0.1 M PBS for 15 min prior to being incubated with rabbit polyclonal c-Fos antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA; no. sc-52) in PBS containing 1% NGS and 0.3% Triton X-100 at 4°C for 48 h (McDougall *et al.* 2004; Jupp *et al.* 2011). Sections were then washed in PBS (3 × 5 min) and incubated with biotinylated goat anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA, USA; 1:500) in PBS containing 1% NGS and 0.3% Triton X-100 for 1 h at room temperature. Following washing (PBS, 3 × 5 min) sections were incubated with streptavidin-conjugated horseradish peroxidase (Vector Laboratories, 1:500) in PBS containing 1% NGS and 0.3% Triton X-100 for 1 h at room temperature. Sections were again washed (PBS, 3 × 5 min) prior to incubation with nickel enhanced 3,3′-diaminobenzidine tetrahydrochloride chromagen (DAB, Sigma-Aldrich, St Louis, MO, USA) solution containing 25% 0.4 M PBS and 0.004% w/v ammonium chloride/ammonium nickel (II) sulfate hexahydrate for 10–15 min, and immunoreactivity was developed by addition of hydrogen peroxide. The reaction was terminated by washing with 0.1 M PBS, and sections were subsequently slide mounted with 0.5% gelatin and allowed to air dry overnight. Once dried, the slides were serially dehydrated, cleared and cover-slipped as described above.

Fos quantification

Sections from each treatment group were processed simultaneously for each discrete brain region. Tissue was visualised using a Leica DM LB-2 microscope and Fos-positive cell counts were performed on one to two sections per region of interest. Care was taken to ensure sections were matched at the same anatomical level for each mouse, and the investigator was blinded to the experimental groups.

The brain regions examined included the orbitofrontal cortex (OFC; bregma 2.10); prelimbic cortex, infralimbic cortex and anterior cingulate cortex (PL, IL and AC, respectively; bregma 1.54); nucleus accumbens core and shell (NAc; bregma 1.18); bed nucleus of the stria terminalis and ventral pallidum (BNST and VP, respectively; bregma 0.14); paraventricular nucleus of the thalamus (PVA; bregma −0.34); lateral habenula (bregma −1.46); lateral hypothalamus (bregma −0.58 and −1.58); paraventricular nucleus of the hypothalamus (PVN; bregma −0.70); basolateral amygdala and central nucleus of the amygdala (BLA and CeA, respectively; bregma −1.22); CA1/2 region of the hippocampus, CA3 region of the hippocampus and dentate gyrus region of the hippocampus (CA1/2, CA3 and DG, respectively; bregma −1.46); periaqueductal grey (PAG; anterior bregma −3.28, posterior bregma –4.26); substantia nigra pars compacta, substantia nigra pars reticulata and ventral tegmental area (SNc, SNr and VTA, respectively; bregma −3.4); dorsal raphe (bregma −4.60); and locus coeruleus (LC; bregma – 5.34).

Statistical analyses

All statistical analyses were performed using SigmaStat 3.5 software. For morphine and sucrose self-administration

and cue-induced reward seeking data, two-way repeated measures ANOVAs were used with lever identity (active, inactive) and reinforcer identity (sucrose, morphine) as factors. Student–Newman–Keuls (SNK) *post hoc* analyses were performed when appropriate. For the immunohistochemical data, statistical analyses were performed using two-way ANOVA tests with reinforcer (sucrose, morphine) and 'relapse' test (yes, no) as factors, and Bonferroni t tests for multiple comparisons were performed where appropriate. For the NAc core and CeA the analyses indicated trends toward an interaction that may have been obscured by low power and strong main effects. Thus to further investigate possible group differences in these regions, one-way ANOVAs with Bonferroni t tests were performed. If the data were not normally distributed transformations were performed prior to analysis. For the DG the data could not be normalised, and therefore this region was analysed by Kruskal–Wallis one-way ANOVA on ranks. Differences were deemed statistically significant if $P < 0.05$.

Results

Stable self-administration of both sucrose and morphine in CD1 mice

As shown in Fig. 1*A* and *B*, CD1 mice readily self-administered both sucrose and morphine and maintained responding for both reinforcers over the self-administration period. This robust self-administration is demonstrated by the strong preference for the active lever over the inactive lever in the case of both reinforcers (see Fig. 1*C*). Analysis of lever-pressing data by two-way repeated measures ANOVA revealed a significant interaction between reinforcer and lever identity ($F_{(1,38)} = 227.105$, $P < 0.001$). SNK *post hoc* analyses revealed that for both reinforcers responding on the active lever was significantly higher than responding on the inactive lever (sucrose: $q = 32.803$, $P < 0.001$; morphine: $q = 3.563$, $P < 0.05$). *Post hoc* analyses also revealed that responding for sucrose was significantly greater than responding for morphine ($q = 29.92$, $P < 0.001$).

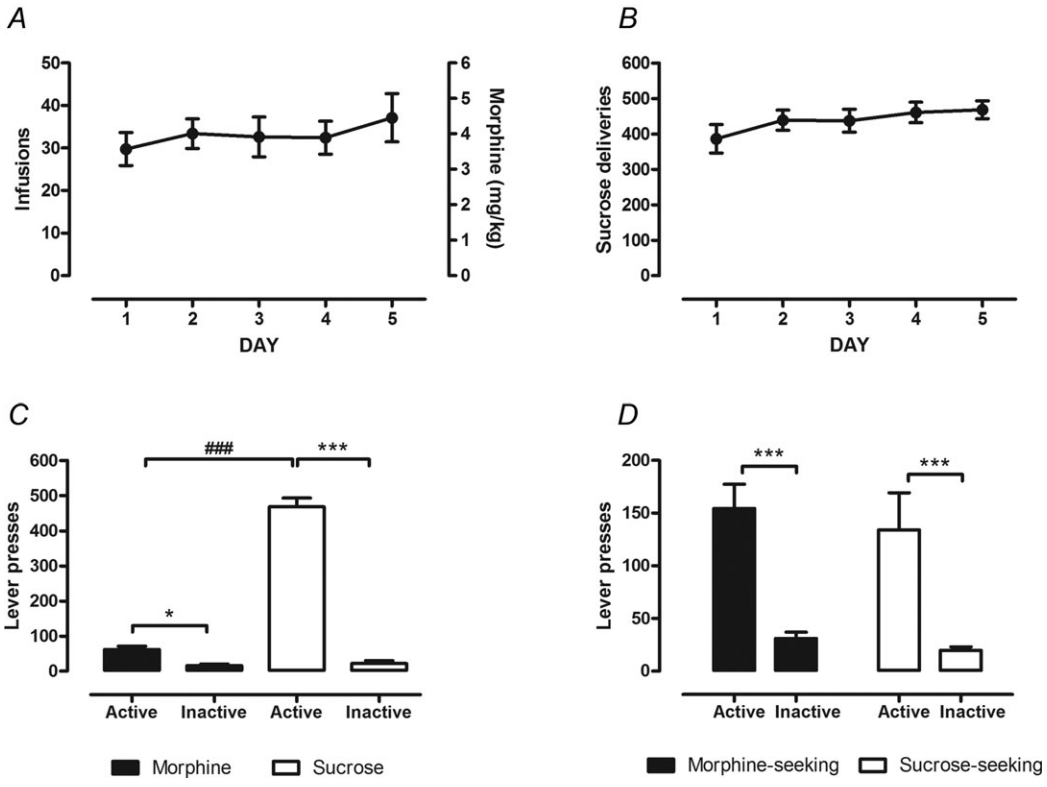

Figure 1. Self-administration and reward seeking in CD1 mice
Data expressed as means (±SEM) for *A–D*. *A*, average of the total number of infusions administered, amount of morphine received (0.1 mg kg^{-1} per infusion) over 5 days of morphine self-administration. *B*, average of the total number of sucrose deliveries over 5 days of sucrose self-administration. *C*, aggregate data for both morphine and sucrose self-administration; $^{*}P < 0.05$, $^{***}P < 0.001$ active lever compared to inactive lever, $^{\#\#\#}P < 0.001$ active lever presses for one treatment group compared to the other (RM two-way ANOVA). *D*, cue-induced reward seeking in mice that have self-administered morphine ($n = 17$) or sucrose ($n = 14$). Total active lever presses as compared to inactive lever presses during the reward seeking session (cues present but no drug infusions); $^{***}P < 0.001$ as compared to inactive lever presses (two-way repeated measures ANOVA).

Similarly robust reward seeking in response to cues associated with both drug and natural reward is observed after a period of abstinence

As shown in Fig. 1*D*, mice exhibited robust reward seeking behaviour in response to cues associated with both drug and natural reward. Reward seeking behaviour was operationally defined as responses on the previously active lever in the absence of reinforcement. Analysis of reward seeking data by two-way repeated measures ANOVA revealed that responding on the active lever was similarly robust for both reinforcers with a main effect of lever identity but not reinforcer ($F_{(1,61)} = 36.333$, $P < 0.001$) and there was no interaction between these two factors. Thus, despite sucrose mice demonstrating increased reward responding during the self-administration sessions compared to morphine mice, no difference was found between the two groups during cue-induced reward seeking.

Fos activation in cortical regions

A number of cortical regions were examined for Fos-immunoreactivity, including the AC, OFC, PL and IL. Statistical analysis by two-way ANOVA revealed a main effect of relapse test for both the AC ($F_{(1,14)} = 8.9$, $P = 0.003$, Fig. 2*A*) and OFC ($F_{(1,14)} = 13$, $P = 0.01$, Fig. 2*B*) and no interaction, indicating increased Fos expression for both morphine and sucrose 'relapse' groups compared to withdrawal. There were no significant differences in Fos expression between the groups in the IL or PL (Table 1).

Fos activation in striatal-limbic circuitry

As can be seen in Fig. 3*A*, both 'relapse' groups exhibited robust Fos expression in the NAc shell, and statistical analysis revealed a significant main effect of relapse test ($F_{(1,14)} = 10.5$, $P = 0.006$) and no interaction. In the NAc core, two-way ANOVA revealed a significant main effect of relapse test ($F_{(1,14)} = 6.5$, $P = 0.023$) and a trend towards an interaction. One-way ANOVA with Bonferroni *t* test indicated more pronounced activation in the morphine 'relapse' group compared to sucrose 'relapse' mice ($t = 2.4$, $P = 0.04$; Fig. 3*B*). The lateral habenula exhibited significant Fos activation in both relapse groups, with a significant main effect of relapse test ($F_{(1,14)} = 8$, $P = 0.013$) and no interaction (Fig. 3*C*). In the VP, there were no significant differences in Fos activation between groups (Table 1).

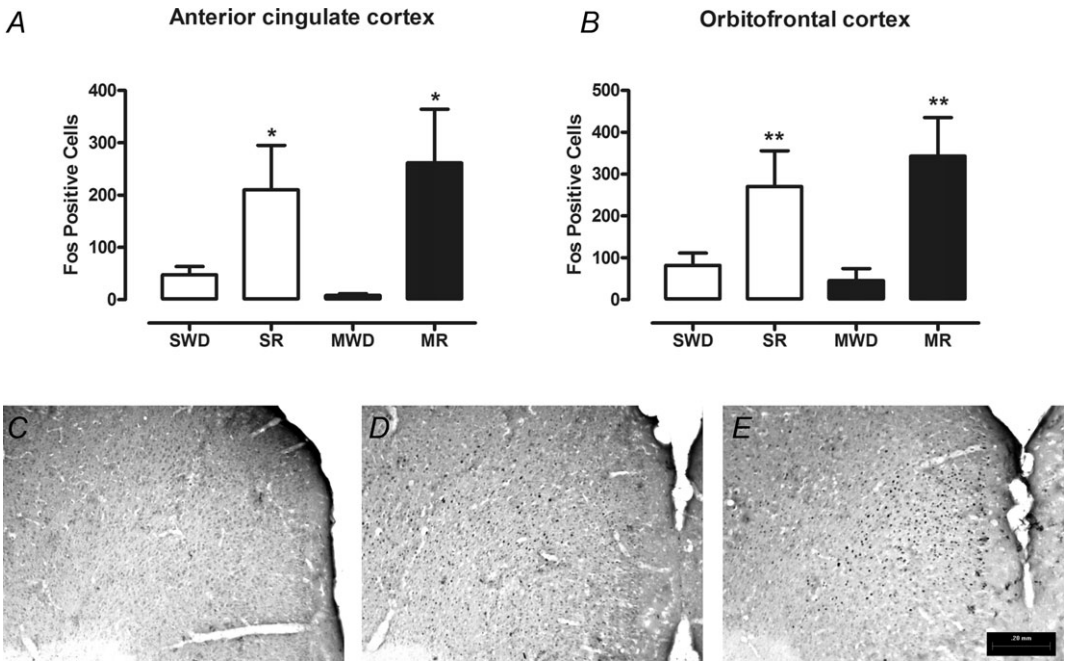

Figure 2. Quantification of Fos-positive nuclei in cortical regions
A and *B*, quantification of Fos-positive nuclei in anterior cingulate cortex (AC; bregma 1.54) (*A*) and orbitofrontal cortex (OFC; bregma 2.10) (*B*) for sucrose withdrawal (SWD), sucrose 'relapse' (SR), morphine withdrawal (MWD) and morphine 'relapse' (MR) mice ($n = 4$–5 per group). Data are expressed as mean number of Fos-positive nuclei per group (± SEM); $^*P < 0.05$, $^{**}P < 0.01$, as compared to withdrawal (two-way ANOVA). *C*–*E*, representative micrographs of Fos-positive neurons in the anterior cingulate from morphine withdrawal (*C*), morphine 'relapse' (*D*) and sucrose 'relapse' (*E*) mice. Scale bar = 0.2 mm

Table 1. Mean (±SEM) Fos positive cells per region of interest for sucrose withdrawal (SWD), sucrose 'relapse' (SR), morphine withdrawal (MWD) and morphine 'relapse' (MR) mice (n = 4–5 per group)

Brain region	SWD ($n = 5$)	SR ($n = 4$)	MWD ($n = 4$)	MR ($n = 5$)
Infralimbic cortex	33.6 (±8.1)	100.3 (±59.1)	15.5 (±2.0)	26.9 (±8.6)
Prelimbic cortex	100.2 (±15.6)	196.9 (±89.5)	21.4 (±9.0)	110.1 (±38.6)
CA1/CA2	6.0 (±0.4)	29.3 (±19.3)**	2.0 (±1.2)	70.2 (±40.8)**
CA3	1.2 (±0.5)	15.8 (±6.1)**	3.0 (±1.7)	25.0 (±13.7)**
Dentate gyrus	1.2 (±0.6)	14.8 (±9.8)	4.3 (±1.7)	16.4 (±10.5)
PVA	12.4 (±6.4)	79.4 (±46.6)	17.8 (±7.1)	42.0 (±13.0)
Posterior PAG	58.8 (±21.2)	177.8 (±90.4)*	97.3 (±87.9)	137.6 (±71.1)*
PVN	4.0 (±0.9)	14.8 (±9.1)	6.8 (±1.8)	10.6 (±3.7)

CA1/CA2, CA1/CA2 region of the hippocampus; CA3, CA3 region of the hippocampus; DG, dentate gyrus; PVA, periventricular nucleus of the thalamus; anterior PAG, anterior periaqueductal grey; PVN, paraventricular nucleus of the hypothalamus. *$P < 0.05$, **$P < 0.01$ as compared to withdrawal.

Quantified Fos expression in the BLA, CeA and BNST is also shown in Fig. 3. In the BLA, two-way ANOVA revealed a main effect of relapse test ($F_{(1,14)} = 75.8$, $P < 0.001$), a main effect of reinforcer ($F_{(1,14)} = 28.1$, $P < 0.001$) and a significant interaction between these two factors ($F_{(1,14)} = 9.6$, $P = 0.008$). *Post hoc* analysis revealed increased Fos activity in the morphine 'relapse' group compared to morphine withdrawal ($t = 8.3$, $P < 0.001$) and sucrose 'relapse' mice ($t = 5.9$, $P < 0.001$) and in sucrose 'relapse' mice compared to sucrose withdrawal ($t = 4.0$, $P < 0.001$; Fig. 3*D*). In the CeA, two-way ANOVA revealed a main effect of relapse test ($F_{(1,14)} = 21.9$, $P < 0.001$) and a trend towards an interaction ($P = 0.067$). Further analysis by one-way ANOVA indicated increased Fos activation in the morphine 'relapse' group compared to sucrose 'relapse' mice ($t = 2.8$, $P = 0.042$; Fig. 3*E*). In the BNST, two-way ANOVA revealed a main effect of relapse test ($F_{(1,14)} = 6.8$, $P = 0.021$) and no interaction (Fig. 3*F*).

The CA1/CA2 region of the hippocampus was activated in both 'relapse' groups with two-way ANOVA indicating a main effect of relapse test ($F_{(1,14)} = 15$, $P = 0.002$) and no interaction (Table 1). Similarly, two-way ANOVA revealed a main effect of relapse test for the CA3 region of the hippocampus ($F_{(1,14)} = 14.1$, $P = 0.002$) and no interaction (Table 1). There were no significant differences in Fos expression between the groups in the DG (Table 1).

The SN showed a different pattern of activation in the pars reticulata compared to the pars compacta. In the SNc, analysis by two-way ANOVA revealed a main effect of relapse test ($F_{(1,14)} = 43.5$, $P < 0.001$) and no interaction, indicating increased Fos activation in both 'relapse' groups compared to withdrawal (Fig. 4*A*). In the SNr, Fos activation was greatest in the morphine 'relapse' group. Analysis by two-way ANOVA indicated a significant main effect of relapse test ($F_{(1,14)} = 73.5$, $P < 0.001$) and a significant interaction ($F_{(1,14)} = 12.1$, $P = 0.004$). *Post hoc* analysis revealed increased Fos activation in sucrose 'relapse' mice compared to sucrose withdrawal ($t = 3.6$, $P = 0.003$) and in morphine 'relapse' mice compared to both morphine withdrawal ($t = 8.5$, $P < 0.001$) and sucrose 'relapse' ($t = 2.9$, $P = 0.011$; Fig. 4*B*). In the VTA, analysis by two-way ANOVA revealed a significant main effect of relapse test ($F_{(1,14)} = 61.0$, $P < 0.001$) and no interaction, indicating activation of the VTA in both relapse groups compared to withdrawal (Fig. 4*C*).

Fos activation in hypothalamic nuclei and brainstem

As can be seen in Fig. 5*A*, particularly strong Fos immunoreactivity was evident in the lateral hypothalamus for both 'relapse' groups, and analysis by two-way ANOVA revealed a significant main effect of relapse test ($F_{(1,14)} = 41.3$, $P < 0.001$) and no interaction. The dorsal raphe was the only region found to be activated in the sucrose 'relapse' group only. Analysis by two-way ANOVA indicated a main effect of relapse test ($F_{(1,14)} = 11.2$, $P = 0.005$) and a significant interaction ($F_{(1,14)} = 5.6$, $P = 0.024$). Subsequent *post hoc* analysis revealed increased Fos activity in the sucrose 'relapse' group compared to both sucrose withdrawal ($t = 4$, $P = 0.001$) and morphine 'relapse' mice ($t = 2.7$, $P = 0.016$). Two-way ANOVA also revealed a main effect of relapse test for the anterior PAG ($F_{(1,14)} = 16.3$, $P < 0.001$; Fig. 5*C*), posterior PAG ($F_{(1,14)} = 5.7$, $P = 0.032$; Table 1) and LC ($F_{(1,14)} = 15.6$, $P < 0.001$; Fig. 5*D*) and no interaction, indicating increased Fos activation in these regions in the 'relapse' groups compared to withdrawal. No statistically significant differences were observed between groups in the PVA or PVN (Table 1).

Discussion

This is the first comprehensive brain mapping study to compare the neural substrates that underlie opiate seeking

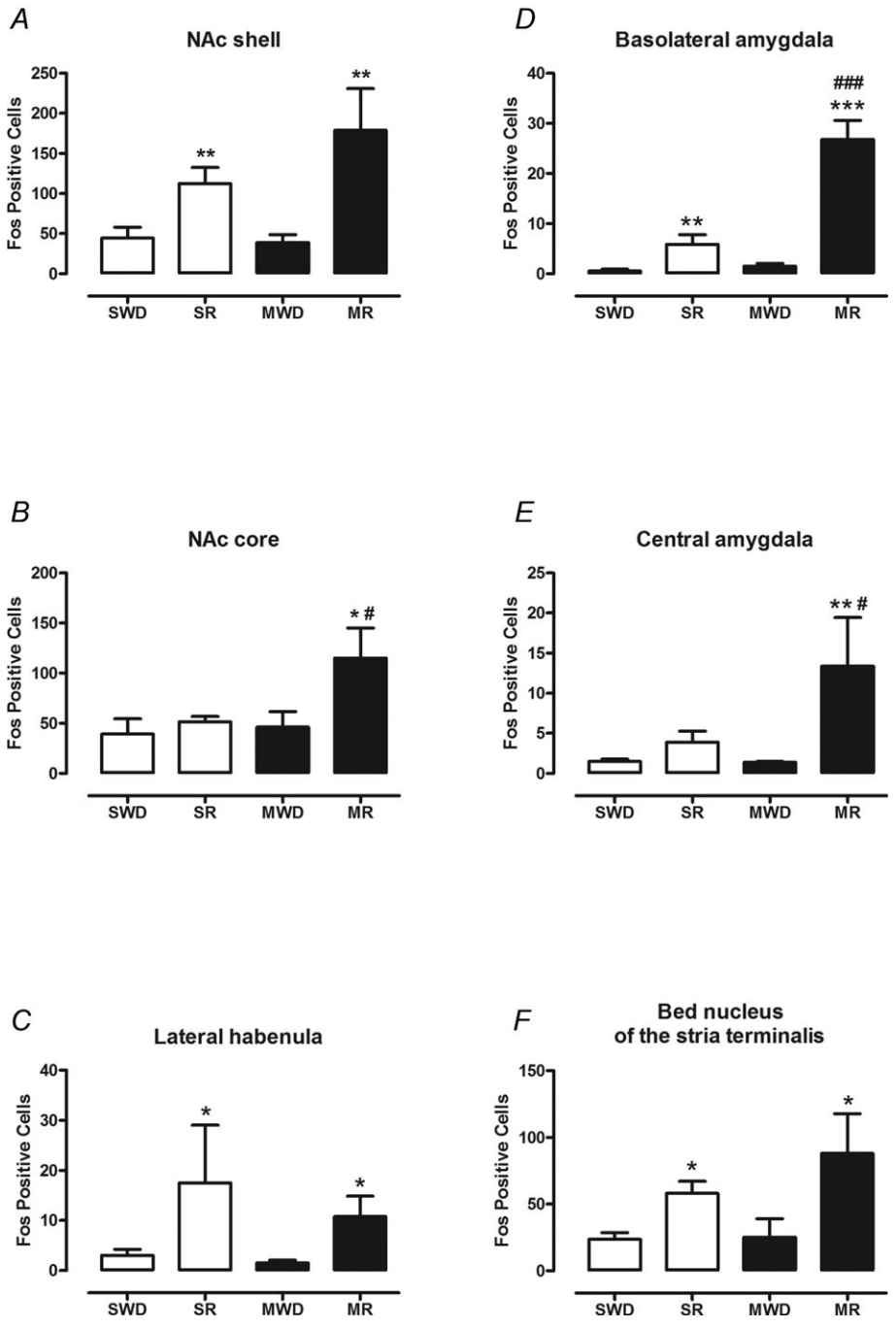

Figure 3. Quantification of Fos-positive nuclei in limbic regions
A–C, quantification of Fos-positive nuclei in nucleus accumbens shell (NAc shell, bregma 1.18) (*A*), nucleus accumbens core (NAc core, bregma 1.18) (*B*), and lateral habenula (bregma −1.46) (*C*) for sucrose withdrawal (SWD), sucrose 'relapse' (SR), morphine withdrawal (MWD) and morphine 'relapse' (MR) mice ($n = 4$–5 per group). Data are expressed as mean number of Fos-positive nuclei per group (± SEM); $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to withdrawal, $\#P < 0.05$ as compared to sucrose relapse group (two-way ANOVA or one-way ANOVA with Bonferroni *t* tests). *D–F*, quantification of Fos-positive nuclei in basolateral amygdala (BLA, bregma −1.22) (*D*), central amygdala (CeA; bregma −1.22) (*E*) and bed nucleus of the stria terminalis (BNST; bregma 0.14) (*F*) for sucrose withdrawal (SWD), sucrose 'relapse' (SR), morphine withdrawal (MWD) and morphine 'relapse' (MR) mice ($n = 4$–5 per group). Data are expressed as mean number of Fos-positive nuclei per group (±SEM); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to withdrawal, $\#P < 0.05$, $\#\#\#P < 0.001$ as compared to sucrose relapse group (two-way ANOVA or one-way ANOVA with Bonferroni *t* tests).

vs. natural reward seeking following abstinence in mice. There is a clear need for this work given the increasing use of genetically modified mice in addiction research. We have found that cue-induced reward seeking after a period of abstinence is similarly robust for both natural and drug reinforcers. In addition, a number of brain regions were activated in response to both morphine and sucrose seeking, including the AC, OFC, NAc shell, BNST, hippocampus, SNc, VTA, PAG, LC and lateral habenula. The dorsal raphe was the sole structure found to be activated only in sucrose 'relapse' mice. Structures that exhibited stronger activation in the morphine 'relapse' group compared to the sucrose 'relapse' group included the NAc core, BLA, CeA and SNr. Broadly speaking, this pattern of activation supports a cortico-striatal limbic circuit driving opiate seeking behaviour, and we have identified some additional structures that warrant further investigation.

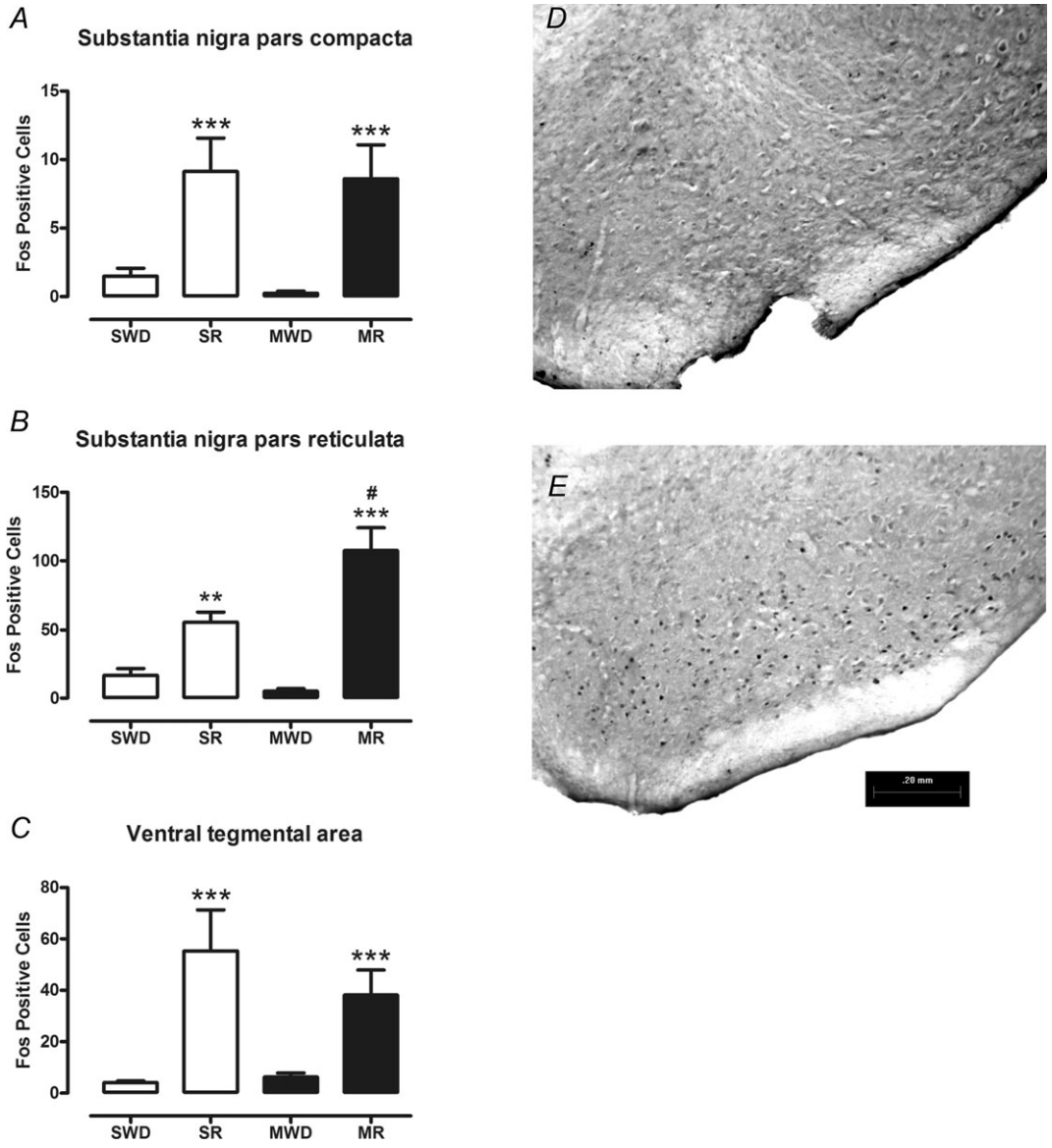

Figure 4. Quantification of Fos-positive nuclei in the midbrain
A–C, quantification of Fos-positive nuclei in substantia nigra pars compacta (SNc; bregma −3.4) (*A*), substantia nigra pars reticulata (SNr; bregma −3.4) (*B*), and ventral tegmental area (VTA; bregma −3.4) (*C*) for sucrose withdrawal (SWD), sucrose 'relapse' (SR), morphine withdrawal (MWD) and morphine 'relapse' (MR) mice ($n = 4$–5 per group). Data are expressed as mean number of Fos-positive nuclei per group (±SEM); $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to withdrawal, $\#P < 0.05$, as compared to sucrose 'relapse' group (two-way ANOVA with Bonferroni *t* tests). Representative micrographs of Fos-positive neurons in the SN from morphine withdrawal (*D*) and morphine 'relapse' mice (*E*). Scale bar = 0.2 mm.

Activation of cortical regions

The AC and OFC both exhibited significant Fos activation in the morphine and sucrose 'relapse' groups. Increased expression of Fos and other IEGs has previously been demonstrated in cortical regions in response to cue-induced reinstatement of opiate seeking in rats (Koya *et al.* 2006), as well as exposure to an environment associated with non-contingent opiate administration (Schroeder *et al.* 2000; Schroeder & Kelley, 2002). Fos expression is also enhanced in the AC and OFC in response to cocaine seeking in rats following abstinence (Neisewander *et al.* 2000; Zavala *et al.* 2007; Zavala *et al.*

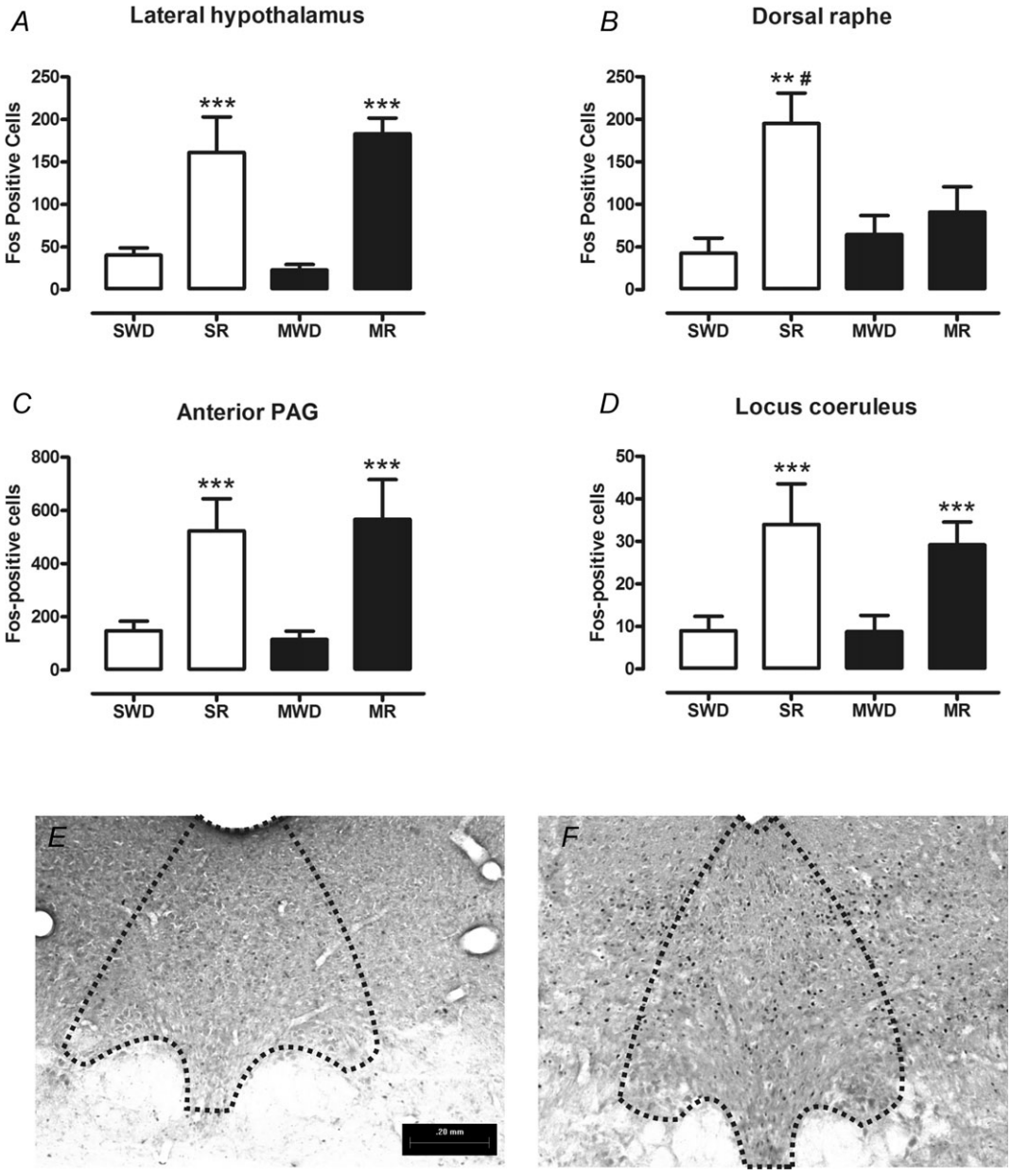

Figure 5. Quantification of Fos-positive nuclei in brainstem and hypothalamic regions
A–D, quantification of Fos-positive nuclei in lateral hypothalamus (bregma −0.58 and −1.58) (*A*), dorsal raphe (bregma −4.60) (*B*), anterior periaqueductal grey (anterior PAG; bregma −3.28) (*C*), and locus coeruleus (LC; bregma −5.34) (*D*) for sucrose withdrawal (SWD), sucrose 'relapse' (SR), morphine withdrawal (MWD) and morphine 'relapse' (MR) mice ($n = 4$–5 per group). Data are expressed as mean number of Fos-positive nuclei per group (±SEM); $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to withdrawal, $\#P < 0.05$ as compared to sucrose 'relapse' (two-way ANOVA with Bonferroni *t* tests). Representative micrographs of Fos-positive neurons in the dorsal raphe from (*E*) sucrose withdrawal and (*F*) sucrose relapse mice. Scale bar = 0.2 mm

2008), indicating that activation of these regions is not specific to opiate seeking, and likely represents a putative common pathway involved in cue-induced drug seeking.

The lack of significant activation in the IL and PL in this study is interesting given that these regions have been reported to exhibit enhanced Fos expression in response to reinstatement of opiate seeking, and exposure to opiate-associated cues in rats (Schroeder *et al.* 2000; Schroeder & Kelley, 2002; Schmidt *et al.* 2005; Bossert *et al.* 2011). Activation of the PL and IL has also been demonstrated following cue-induced cocaine seeking in rats (Zavala *et al.* 2007, 2008) and more recently in response to reinstatement of cocaine seeking in mice (Ziolkowska *et al.* 2011). These differences are possibly due to lack of prior extinction training in the present study, and may also be due to varying sensitivity in the methods used to examine neuronal activation, time course for optimal protein expression and/or biological variability. It should be noted that in the case of morphine seeking, there was a strong tendency for activation of the PL (and also to a lesser extent for sucrose seeking), in line with behavioural studies in rats and mice. On balance, the present data do not at all preclude a role for PL and/or IL in cue-driven initial reward seeking in mice.

Importantly, there are remarkable similarities between the findings of the present study and human imaging data. Activation of both the AC and OFC has been observed in drug addicted individuals upon exposure to drug-associated cues that precipitate craving (Goldstein & Volkow, 2002; Kalivas & Volkow, 2005), and this degree of activation correlates with the intensity of self-reported craving (Volkow *et al.* 1999, 2003; Wexler *et al.* 2001). Activation of the AC in the sucrose 'relapse' group is also consistent with a recent report that found increased activation of the AC in food addicted individuals in response to food anticipation (Gearhardt *et al.* 2011).

Activation of amygdaloid nuclei

The BLA plays a critical role in cue conditioning, and the importance of this structure in cue-induced reinstatement of drug seeking is well established (Fuchs & See, 2002; McLaughlin & See, 2003; Rogers *et al.* 2008). Enhanced Fos expression has been observed in both the BLA and CeA in response to cue-induced cocaine seeking during abstinence (Neisewander *et al.* 2000; Zavala *et al.* 2007) and following a morphine-conditioned place preference (CPP) test (Harris & Aston-Jones, 2003). The findings of the present study support a role for these structures in cue-induced opiate seeking, with activation of both the BLA and CeA in the morphine 'relapse' group. This is also consistent with human imaging studies which demonstrate activation of the amygdala in drug addicted individuals upon presentation of drug-associated cues (Childress *et al.* 1999; Kilts *et al.* 2001). The BLA and CeA were also activated in sucrose 'relapse' mice, though to a lesser extent. This is in agreement with research that has shown Fos induction in the BLA in response to food-induced CPP (Harris & Aston-Jones, 2007). The CeA has also been implicated in incubation of craving, a phenomenon where craving for drugs increases over time during abstinence (Pickens *et al.* 2011). Incubation of craving for both cocaine and opiates has been specifically linked to ERK signalling in the CeA (Lu *et al.* 2005; Li *et al.* 2008). Activation of the CeA in the morphine relapse group is consistent with increased ERK signalling in this region following abstinence.

The CeA, BNST and NAc shell are all components of the extended amygdala, a composite structure which is uniquely positioned to encompass both reward and stress circuitry (Alheid & Heimer, 1988). Reversible inactivation of the BNST or CeA attenuates stress-induced reinstatement of heroin seeking (Shaham *et al.* 2000), and interruption of the CRF-containing pathway from the CeA to the BNST reduces stress-induced reinstatement of cocaine seeking (Erb *et al.* 2001). While the BNST has traditionally been implicated in stress-induced reinstatement (Erb & Stewart, 1999; Shaham *et al.* 2000; McFarland *et al.* 2004), it has recently also been identified for a role in cue-induced reinstatement of cocaine seeking (Buffalari & See, 2011), and activation of this region in the morphine 'relapse' group suggests that it may also be involved in cue-induced opiate seeking. Enhanced activation of this stress circuitry in the morphine 'relapse' group compared to the sucrose 'relapse' group suggests that drug-associated may cues evoke a stronger stress response than cues associated with a natural reward.

Support for a putative final common pathway

The NAc core receives input from the PFC and is hypothesised to be a component of a final common pathway that drives drug seeking behaviour (Kalivas & Volkow, 2005). While most of the evidence for this has accumulated from research into cocaine seeking, activation of the PFC and the NAc core in morphine 'relapse' mice supports a role for this circuit in cue-induced opiate seeking. Increased activation of the SN in morphine 'relapse' mice is also consistent with enhanced NAc core outflow to this region. A critical role for this circuit in opiate seeking has been supported by pharmacological studies demonstrating that inactivation of the PFC, NAc core, or SN attenuates cue and primed reinstatement of heroin seeking in rats (Rogers *et al.* 2008). Only some of these structures were robustly activated in sucrose 'relapse' mice, suggesting that this pathway cannot necessarily be generalised to food seeking.

While there is strong evidence for a critical role for the NAc core in cue-induced opiate seeking, the

role of the NAc shell is less clear. In the present study the NAc shell exhibited significant Fos activation in both 'relapse' groups. The NAc shell has connections with the hypothalamus, BLA and VTA and is involved in the regulation of ingestive behaviours and appetitive motivation (Kelley, 2004). Enhanced Fos expression is observed in the NAc shell after exposure to morphine-associated contextual cues (Schroeder *et al.* 2000), and following context-induced reinstatement of alcohol seeking (Hamlin *et al.* 2007). In line with this, research in rats has revealed a critical role for the NAc shell in context-induced reinstatement of heroin (Bossert *et al.* 2006; Bossert *et al.* 2007), alcohol (Chaudhri *et al.* 2009) and cocaine seeking (Fuchs *et al.* 2008). However, pharmacological lesioning of the NAc shell does not significantly attenuate cue-induced heroin (Rogers *et al.* 2008) or cocaine seeking (Fuchs *et al.* 2004), suggesting that a critical role for this structure may be specific to context-induced reinstatement. This is consistent with literature suggesting that the NAc shell regulates drug seeking behaviour driven by spatial and contextual information, whereas the NAc core is more important for drug seeking under the control of discrete cues (Bossert *et al.* 2007; Ito *et al.* 2008; Chaudhri *et al.* 2010).

Activation of the LC, PAG, hippocampus and lateral habenula in both 'relapse' groups

The LC and PAG showed significant activation in both 'relapse' groups. These structures have both been implicated in opiate withdrawal (Rasmussen *et al.* 1990; Punch *et al.* 1997), and exhibit Fos activation in response to acute naloxone precipitated withdrawal (Frenois *et al.* 2005). Withdrawal from opiates would not explain their activation in the present study, however, as drug naive sucrose 'relapse' mice also exhibited Fos activation and the morphine withdrawal mice did not. The LC contains one of the largest populations of noradrenergic neurons in the brain and is involved in stress and arousal (Berridge & Waterhouse, 2003). Consistent with its activation in the 'relapse' groups there is a potential role for the LC in information processing concerning reward expectancy and the incentive value of a conditioned stimulus (Bouret & Sara, 2004). The PAG has traditionally been associated with defensive behaviour and emotional coping to stress (Carrive, 1993; Keay & Bandler, 2001) and more recently the anterior PAG has been implicated in motivation to hunt and forage (Sukikara *et al.* 2006; Mota-Ortiz *et al.* 2009).

Fos induction in hippocampal regions (CA1/CA2 and CA3) was also increased in both morphine 'relapse' and sucrose 'relapse' mice. With respect to cocaine, Fos expression is enhanced in hippocampal regions in response to cue-induced cocaine seeking following both abstinence and extinction in rats (Neisewander *et al.* 2000; Zavala *et al.* 2007); however, no change in hippocampal expression of IEGs has also been reported following reinstatement of cocaine seeking in mice (Ziolkowska *et al.* 2011). To the best of our knowledge the present study is the first to examine Fos expression in the hippocampus in response to cue-elicited opiate seeking, and further studies utilising pharmacological inactivation techniques would be useful for determining the importance of this region in relapse to opiate seeking.

The lateral habenula is another structure that exhibited significant activation in both 'relapse' groups. The lateral habenula serves as a convergence point for limbic and basal ganglia circuits (Hikosaka *et al.* 2008), making it well positioned to influence reward-related behaviours. Increased Fos expression is observed in the lateral habenula in response to cue-induced reinstatement of heroin seeking (Zhang *et al.* 2005), and also upon exposure to a cocaine-paired environment (Brown *et al.* 1992; Franklin & Druhan, 2000). Fos expression is also increased in the lateral habenula following reinstatement of a cocaine CPP (Brown *et al.* 2010), and in rats that exhibit high levels of cue-induced reinstatement of cocaine seeking in an operant paradigm (James *et al.* 2011). Consistent with increased Fos expression in the sucrose 'relapse' group, Fos activation in the lateral habenula is also observed upon presentation of a food-associated cue that has been attributed to incentive salience (Flagel *et al.* 2011).

The lateral hypothalamus exhibits strong activation in both relapse groups

Activation of the lateral hypothalamus was particularly strong in both morphine 'relapse' and sucrose 'relapse' mice. The lateral hypothalamus has dense connections with many limbic structures including the VTA, striatum, NAc and amygdala (Saper *et al.* 1979; Zahm & Heimer, 1993; Petrovich *et al.* 1996) and as such it is well positioned to exert modulation upon the dopaminergic system to regulate motivated behaviours. Cue-induced reinstatement of alcohol seeking (Dayas *et al.* 2008) and context induced reinstatement of sucrose (Hamlin *et al.* 2006), alcohol (Hamlin *et al.* 2007) and cocaine seeking (Hamlin *et al.* 2008) all increase Fos expression in lateral hypothalamic neurons. Inactivation of the lateral hypothalamus via infusion of GABA agonists abolishes context-induced reinstatement of sucrose and alcohol seeking (Marchant *et al.* 2009), demonstrating a critical role for this structure in context-induced reward seeking.

There are a number of neuropeptides expressed in the lateral hypothalamus including orexin and melanin-concentrating hormone (MCH) (Bittencourt *et al.* 1992; Sakurai *et al.* 1998). The orexin system was

traditionally implicated in wakefulness and arousal, but more recently has been shown to influence both food and drug seeking (Harris *et al.* 2005, 2007; Lawrence *et al.* 2006). Orexin neurons exhibit Fos activation in response to contextual cocaine seeking (Hamlin *et al.* 2008), and are also activated by cues associated with drugs of abuse, food and alcohol (Harris *et al.* 2005). Orexin neurons directly excite VTA neurons (Narita *et al.* 2006), which is consistent with activation of the VTA in both 'relapse' groups in the present study. Orexin neurons also project to the LC and dorsal raphe, both of which were activated in sucrose 'relapse' mice (Peyron *et al.* 1998; Nambu *et al.* 1999). The neuropeptide MCH has also been implicated in cue-induced and drug-primed reinstatement of cocaine seeking (Chung *et al.* 2009). Further studies would be required to confirm the identity of the neurons that are activated in the lateral hypothalamus in response to cue-induced reward seeking under the current paradigm.

Activation of the dorsal raphe is specific to sucrose relapse mice

The dorsal raphe, which contains one of the largest populations of serotonergic neurons in the brain was the only structure activated specifically in sucrose 'relapse' mice. There is evidence that serotonergic transmission from the dorsal raphe to the ventromedial hypothalamus contributes to the regulation of feeding (Ohliger-Frerking *et al.* 2002), and Fos expression in the dorsal raphe is enhanced in response to food anticipation and searching (Takase & Nogueira, 2008). Recently, it has been demonstrated that serotonergic neurons in the dorsal raphe exhibit increased firing when animals are forced to wait for delivery of food and water rewards in response to a conditioned tone (Miyazaki *et al.* 2011). Our findings are thus consistent with activation of the dorsal raphe in response to cue-conditioned expectancy of a food reward.

Conclusions

The findings of the present study broadly support a cortico-striatal limbic circuit driving opiate seeking following abstinence, and in addition we have identified a number of neuroanatomical substrates outside this established motivational circuitry that may contribute to cue-induced reward seeking. This highlights the importance of examining a wide range of structures in brain mapping studies, not just those traditionally implicated in drug seeking behaviour. These findings are important for guiding future research to functionally test involvement of these regions. We have also identified putative circuitry that is common to morphine- and sucrose seeking, as well as that which is specific to one or other reinforcer. Importantly, there is considerable overlap with brain regions that have been implicated in drug craving in human imaging studies. Accordingly, these data support an abstinence model of relapse as a relevant preclinical model to study drug seeking in mice.

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Author contributions

Experiments were conceived and designed by A.J.L. with contributions from R.M.B., H.B.M. and J.L.S. Collection, analysis and interpretation of data were performed by R.M.B., H.B.M. and A.J.L. The manuscript was drafted by H.B.M. and R.M.B., and revised by A.J.L. and J.L.S. All experiments were performed at the Florey Neuroscience Institutes, Parkville, Vic, Australia, and all authors approved the final version for publication

Acknowledgements

These studies were supported in part by a project grant (566 736) from the NHMRC (Australia) of which R.M.B. is an Early Career Research Fellow (1037 080) and A.J.L. is a Principal Fellow (1020 737). Financial support from the Pratt/Besen Foundations and the Victorian Government's Operational Infrastructure Support Program is gratefully acknowledged.